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13. ABSTRACT

Garter snakes (Thamnophis spp) are capable of overwintering the Western encephalitis (WE) virus and possesses enough virus in the blood to infect a Culex tarsalis mosquito in the spring when these snakes come out of hibernation. These mosquitoes (C. tarsalis) are readily infected by blood meals from infected snakes (10^2 or above virus per ml of blood in the snakes) and infected C. tarsalis mosquitoes readily infect snakes when blood meals are taken. Snakes may exhibit a cyclic viremia, that is positive, then negative, then positive virus in the blood stream. In studies of about 1800 lip tagged snakes, foci of virus may be present in one area, but absent in other areas. Garter snakes wander less than 100 yards from their native area. Snakes have been found naturally infected in nature with WE virus. Climatological conditions determine when mosquitoes will bite snakes in nature, and temperatures between 50 and 65° F appear to be optimum. These temperatures are part of the spring and late summer or early fall weather conditions. Mosquitoes do not bite snakes during the hot months of the year. Snakes infected 2 to 11 days before hibernation will allow a viremia to be carried over the winter months. Snakes infected 19 or more days before they hibernate fail to produce an overwintering viremia, but develop antibody. Thus a sharp drop in temperature in the late summer or early fall will determine the overwintering of virus in snakes that are infected that go into hibernation soon after being infected. Thus virus in these snakes is available the next spring for mosquitoes.

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ECOLOGY AND EXPERIMENTAL EPIDEMIOLOGY OF WESTERN ENCEPHALITIS VIRUS

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From the UNIVERSITY OF UTAH
by

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Principal Investigator

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Ecology and Experimental Epidemiology of Western Encephalitis Virus

The virus of Western encephalitis was isolated by Meyer et al., in 1931, during a severe enzootic of horses in California. Since then major epizootics have been present in horses (176,000 cases - 1937) as well as widespread epidemics in humans (Lennette et al.; McClintock et al.) This virus has not been limited to the Western United States, but is present in the mid-Central states, Eastern states, Canada, and South America.

Several authors have demonstrated that Culex tarsalis mosquito is the principal vector of this disease agent (Kesler; Reeves et al.; Hammon et al.; Reeves and Hammon et al.) and that many species of birds are naturally infected by mosquitoes. (Holden; Kissling et al.; Stamm; Hayes et al.; Reeves et al.; Miles; Stark).

The overwintering mechanism of the WE virus has been postulated by many research workers, but the exact mechanism has evaded attempts to explain the most probable means by which virus overwinters and becomes available to C. tarsalis mosquitoes in the spring.

Birds have received much attention as an overwintering host (Kissling; Reeves et al.; Holden; Stamm; Hayes et al.), but the concensus is that birds do not develop a protracted viremia for over-carry of the virus and that antibody develops fairly rapidly after infection. Birds do,

however, play a major role as virus reservoirs during the mosquito season and undoubtedly play a specific role in endemic and epidemic periods.

Mosquitoes (C. tarsalis) have been considered as overwintering hosts of WE virus, and on a few occasions, virus has been isolated from the vector mosquitoes during the winter months. (Blackmore and Winn; Reeves et al., and Bellamy et al.) However, Bellamy et al., found that mosquitoes must be in a climate in which activity of the insects is possible during the winter months. Rush et al., suggests that mosquitoes taking a blood meal prior to inactivity in the winter (hibernation?) do not survive the winter months. Bennington et al., also are of the opinion, based on experimental evidence, that mosquitoes which take blood meals prior to winter inactivity fail to survive in cold climate areas.

Other hosts such as mammals have been considered as part of the overwintering mechanism, but various mammals fail to develop viremias for more than a few days then develop antibody. Bats may play a role, particularly hibernating bats, may overwinter virus. LaMotte was able to detect latent Japanese encephalitis virus hibernated for 107 days after infection. Stanton showed that Plecotus towsendii (true hibernator bats) were able to maintain WE virus for 38 days under hibernating conditions, but on returning the bats to room temperature.

they died, presumably of WE infection, since virus was isolated from the blood at this time.

In 1942 Rosenbusch was able to infect snakes with WE virus and these reptiles maintained virus for over four weeks. It was found by Thomas et al.; Thomas and Ekland; and Gebhardt and Hill that garter snakes would hibernate the WE virus for from 70 to 130 or more days, and possessing a sufficient viremia after hibernation to infect C. tarsalis mosquitoes. Karstad (1961) reported arbovirus antibodies in reptiles for EE virus; and in 1962 Karstad experimentally infected turtles, snakes, and lizards with EE virus, and in 1964 WE virus antibody was found in snakes (Spalatin et al.)

In 1964 Gebhardt et al., reported isolation of WE virus from three species of naturally infected snakes. Confirmation of WE virus occurring naturally in snakes in Saskatchewan was reported by Burton et al., in 1966. They also isolated this virus from six frogs and neutralizing antibody in 50 of 179 frogs tested. Dr. Ho W. Lee, in Korea, has isolated Japanese encephalitis from wild caught snakes on two different occasions.

Gebhardt et al., (1966) reported that a single infected mosquito could transmit WE virus to garter snakes. Snakes with a blood virus titer of about 10^2 per ml could infect mosquitoes when these insects take a blood meal from these infected poikilothermic animals.

Methods and Materials

Snakes were captured in various marshy areas in the State of Utah, lip tagged and bled for virus and antibody. Some were returned to their native areas for further study over a four-year period and some were maintained in the laboratory for experimental study.

Culex tarsalis mosquitoes were kept colonized in the laboratory, maintaining egg laying by feeding on baby chicks. Snakes were infected in specially constructed cages, 1 X 1 foot, using vinyl screen with a double thick gauze entry sleeve. Mosquitoes were fed daily a mixture of raisin and Karo syrup placed on gauze pads on the surface of the cage. Plants were kept in the breeding cage for male mosquitoes to obtain plant juices.

For virus isolations, baby mice (4 to 8 days old) were used along with baby chicks less than 12 hours old. Previous data (D. W. Hill) showed that baby chicks were infected with a single plaque forming unit of WE virus. Baby chicks and baby mice were kept in a special isolation room after injection.

Outside cages for snakes and mosquitoes were of two types (1) Two large cages covered with copper fly screen, with gauze sleeve holes to gather mosquitoes and egg rafts, and (2) hardware cloth cage 1/4" mesh to allow only entry of wild mosquitoes. Woodpiles were

maintained in these cages, since it was found that these areas were hiding places for both snakes and mosquitoes and this also maintained close association of snakes and mosquitoes. Boxes were also maintained in these cages with small round entry holes for snakes to hide and rest. Pans of water were kept in these cages for the snakes and for collection of mosquito egg rafts.

Tissue culture techniques have been previously reported (Soc. Exper. Biol. and Med. 123:233-235, 1966.

Snake hibernation was carried out in a dug pit, supplied with a thermocouple to measure temperatures on a 24-hour basis. During the winter months, when outside temperatures varied from 10 to 40° F, the hibernating pit had a fairly constant temperature of 40.1 to 41.9° F. At this temperature, snakes were quite stiff and had to be warmed for bleeding.

Results:

Previous results showed that garter snakes (Thamnophis spp) could overwinter WE virus and in the spring they came out of hibernation, these reptiles showed sufficient virus in the blood to infect the vector mosquito, C. tarsalis. Previous results showed that wild caught snakes are infected in nature and virus may be isolated from these snakes early in the spring suggesting that the virus was overwintered from late summer or early fall.

Snakes captured in some areas are virus or antibody positive, but snakes caught in other areas have had no infection. Over 1800 lip tagged snakes have been studied for 4 years. The above data suggests that virus is endemically present in one area, but may not be present in other areas. This may suggest why virus smolders in one area but not in other areas; thus enabling epidemics to develop in or close to these virus endemic areas, if snake-mosquito-bird population are adequate.

It has been shown that virus infected mosquitoes (C. tarsalis) will readily infect snakes when these take a blood meal from susceptible snakes. A single infected mosquito is capable of infecting a snake. Mosquitoes (C. tarsalis) are infected from snakes which have a viremia in the amount of 1×10^2 to 1.8×10^4 virus particles per ml.

Reasons for snakes in nature which infect mosquitoes in the early spring, and reasons why mosquitoes in the late summer or early fall are infected have been experimentally solved. Over a five-year period, climatological (seasonal) studies have been carried out. During the hot summer months, C. tarsalis mosquitoes do not bite snakes as a blood meal source. Minimum nighttime temperatures of 40 or 45° F are not suitable as temperatures required for C. tarsalis mosquitoes to take blood meals from garter snakes. A few blood meals are taken at temperatures of 46 to 49° F. However, the majority of the biting of snakes by

mosquitoes takes place between 50 and 65° F, nighttime temperature. From 66 to 69° F very little biting takes place. Temperatures of 70° F, or above appear to not invite biting of snakes by mosquitoes. Apparently, warm blooded animals (birds, rodents, horses, man, rabbits, etc.) are preferred blood meal hosts during hot days and nights. The above optimum nighttime temperatures are present both in the spring and late summer or fall months in cold climate areas. It has also been experimentally determined that rock and woodpiles enhance mosquito biting of snakes, apparently as a hiding place for both and close association requirements of insects and snakes. These areas (rock and woodpiles) are also productive areas for catching wild mosquitoes in marshy areas.

Further experimental evidence shows the following reasons for natural overwintering of virus by snakes. Snakes (garter) infected with WE virus, then hibernated 2 and 11 days after infection, develop a viremia that continues from January through April (overwinter the virus). Snakes hibernated 19 and 47 days after infection develop a transient viremia, then develop antibody. Snakes held at room temperature (26 to 30° C) for 171 days develop a transient viremia, then develop antibody. Snakes showing an original 1:10 antibody titer of 50% PFU* reduction will develop a viremia, but snakes having an initial antibody level of 1:100 titer of 50% PFU reduction fail to develop a viremia.

*PFU=plaque forming units

From the above experimental data, if snakes bitten by WE virus infected mosquitoes in the late summer or fall and there is a sudden temperature drop to force the snakes into hibernation from 2 to 11 days after infection, these snakes then maintain a viremia over the winter and serve as virus source for mosquitoes (C. tarsalis) in the spring. If fairly large numbers of snakes are infected, then a good supply of virus will be available in the spring for mosquitoes and a possible epidemic (epizootic) may follow the next summer. If only a few snakes are infected or are immune, or if a sharp temperature drop is not present, then very few snakes will overwinter the virus. These data support the possibility of predicting a Western encephalitis epidemic (epizootic) of endemic virus if endemic virus is present, if climatological data in the late summer or early fall follow the pattern of a sudden, sharp temperature drop. Data is being collected on past naturally occurring human epidemics and horse epizootics to determine if the above experimental data will fit the patterns of human epidemics or horse epizootics.

Attempts to explain the cyclic nature of WE virus in snakes have experimentally eliminated both antibody and interferon production as the underlying mechanism. Antibody can apparently be synthesized during hibernation. Snakes will produce interferon during virus production, both at room temperature and during the first part of hibernation. During the negative phase of virus production, or at a period when no virus is detect-

able, no interferon is produced. When these snakes begin to produce virus for the second time (this varies from 1 to 3 or more months) there is a rise in interferon, then a drop in this substance as the virus in reptile's blood drops.

Summary and Theory of Epidemic and Epizootic Patterns

Garter snakes (Thamnophis spp) are able to hibernate WE virus over the winter months in cold climates. Sufficient virus is present in these poikilothermic animals to enable Culex tarsalis mosquitoes to obtain an infectious blood meal in the spring as specific climatological conditions are met. Thus a reservoir host for the virus to enable overwintering of this infectious agent with no harm to these poikilothermic animals. After some mosquitoes are infected, climatological conditions then indicate warm-blooded animals are the principal blood meal host of these insects. Birds are the most probable host during the summer months and mosquito season; and if large number of birds, both adult and nestling, are infected, then more and more mosquitoes are infected. An endemic foci or pockets of WE virus must be present for the virus-mosquito-bird cycle to become initiated. If large number of mosquitoes and birds become infected, then animals closest to the virus source become infected, e.g., horses. If birds then carry the virus to urban areas, and C. tarsalis mosquitoes are present in large numbers, then there is a possibility of human cases or of even epidemics developing. Adequate mosquito

population are dependent on water supplies (rain, irrigation, junk piles for water to collect, (tires, cans, other vessels), cemetaries with flower vases, irrigation canals, etc.) and adequate temperatures for hatching and about 3 months of continuous sunshine to a level of 90% during the day. When late summer or early fall temperatures drop to a level where mosquitoes will bite snakes, and if infected mosquitoes are present, snakes which may then become infected will then hibernate if a sharp nighttime temperature drop becomes climatologically evident, forcing snakes into hibernation; thus a completion of the cycle, enabling these infected snakes to overwinter the virus. If no sharp drop of temperature is evident, then the infected snakes will develop antibody, and comparatively few snakes will be able to overwinter the virus. Thus large numbers of snakes would have to be caught to find one or two viremic snakes.

Suggested methods of control have been presented to the entomological group. Spraying usually commences in this area in May or June. Spraying of mosquitoes bearing water areas or marsh areas usually is from 10 a. m. until 2 or 3 p. m. Since most mosquitoes rest under leaves, grass blades, rock and woodpiles, they escape the direct spray. Many of these sprays are chemically inert after a few hours. Since the majority of these mosquitoes swarm for fertilization at about dusk, and then go back to their resting areas, they light on the upper surface of leaves, grass, wood or rockpiles to eventually walk to their resting areas. The majority

of the killing efficiency of the chemicals are therefore ineffective by this time. It has been suggested that spraying should start the latter part of March or early April to kill the early emerging mosquitoes and apply such sprays from around 5 or 6 p.m. until dusk. Thus, early hatched mosquitoes (C. tarsalis) would be killed, and later mosquito hatches would be also killed by spraying about every 10 days or 2 weeks at the above evening times. This spraying technique should be started each spring following a sudden late summer or early fall temperature drop.

Publications, Reports, etc., Derived from this ONR Grant

1. Louis P. Gebhardt, G. John Stanton and Stephen de St. Jeor. Transmission of WEE virus to snakes by infected Culex tarsalis mosquitoes. Proc. Soc. Exp. Biol. and Med. 123:233-325, 1966.

2. Louis P. Gebhardt and G. John Stanton. The role of poikilothermic hosts as virus reservoirs. Japanese Jour. Med. Sci. and Biol. 20: 30-34, 1967. (Symposium held in Tokyo, 11th Pacific Science Congress)

3. Louis P. Gebhardt, G. John Stanton and Stephen de St. Jeor. Ecology of Western equine encephalitis in nature. Proc. 21st Annual Meeting of the Utah Mosquito Abatement Society.

4. Stanton, G. J. The role of poikilothermic animals in overwintering Western equine encephalitis virus. 1967. Doctoral Thesis - University of Utah, Department of Microbiology.

5. de St. Jeor, Stephen C. Experimental and natural Western equine encephalitis virus infections in reptiles. 1969. Doctoral Thesis, University of Utah, Department of Microbiology.

6. Stringfellow, Dale A. Interferon production in a poikilothermic model. Master's Thesis, June, 1970.

7. Three progress reports were given: two reports at the Intermountain Meetings, Society of American Microbiologists; one report at Saskatchewan, Annual Meeting of the International Society for Diseases in Nature Transmissible to Man.

8. A paper was given, by invitation, in Tokyo, August 1966, at the 11th Pacific Science Congress on the role of poikilothermic hosts as virus reservoirs.

Publications in Progress

1. L. P. Gebhardt, M. D., Stephen C. de St. Jeor, Ph. D., and G. John Stanton, Ph. D. Temperature dependence on the ecology of Western encephalitis virus. To be submitted in June 1970 for publication. Final draft being typed.

2. L. P. Gebhardt and Dale A. Stringfellow. Interferon production in a poikilothermic model. Will be submitted for publication about December 1970.

3. Climatological requirements for development of epidemics and epizootics of Western equine encephalitis. Over half of this data already collected. It should be ready for submission for publication by spring of 1971.

4. Use of fluorescent antibody to detect incomplete virus in snakes showing cyclic virus. About a fourth of this data already collected.

The above publications will carry the ONR research grant number.

Assisting in the above research activities were:

1. G. John Stanton, who received his Ph. D. in 1968, and is now an Assistant Professor, University of Texas Medical Branch, Galveston, Texas.

2. Stepehn C. de St. Jeor, who received his Ph. D. in 1969, is a Postdoctoral Fellow, University of Texas, Medical Branch, Galveston, Texas.

3. Dale A. Stringfellow - Graduate Student, part-time, who completed his M. S. Degree, June 1970 on Interferon production in snakes infected with WE virus.

4. Richard Melton - Research Assistant - Graduate Student.

Suggested Research That Should Be Continued On This Problem

(1) More vigorous stidies on bird blood, snake blood, rabbit blood, and mosquitoes for several seasons, beginning the exact time snakes come out of hibernation and follow this research through the summer and early fall. Climatological data should be collected during this period. This would give data when mosquitoes were first infected and give an indication of late summer and early fall snake infections and how bird populations fit the chain of events in the infection and spread of this virus.

(2) Determine snake (garter) hibernation areas and dig snakes out of these hibernating places in areas of known endemic foci of this virus. Snakes should be removed about a month after hibernation, about the middle of natural hibernation period and a few weeks before snakes come out of hibernation.

(3) A thorough study of other viruses, EE, St. Louis and Venezuelan, to determine if poikilothermic animals play a role in natural reservoirs for these viruses. Other hibernating animals than snakes should also be studied.

These data would give a better understanding of the encephalitis problem and suggest methods of prevention of epidemics.

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